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FOREWORD

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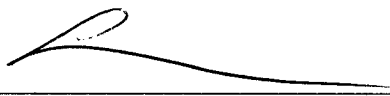
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Introduction:

The principal goal of this project is to understand the transforming growth factor- β (TGF β) receptor signal transduction pathways and the molecular mechanism underlying the regulation of the activity of the TGF β receptor kinases. TGF β could suppress the growth of breast cancer cells both *in vivo* and *in vitro* (1, 2, 3), and this function requires the expression of functional TGF β receptors (2, 3) and downstream signaling molecules (4).

The TGF β family of cytokines has a wide range of biological functions including tumor suppression, extracellular matrix production, embryonic development, and regulation of differentiation(5). These functions are mediated by three specific surface receptors, Types I, II and III, all of which have been cloned (6, 7, 8, 9). The types I and II receptors for TGF β , T β RI and T β RII, are members of the first known receptor serine/threonine kinase family, and share 40% homology between their kinase domains. T β RII contains an extracellular domain which binds TGF β , a transmembrane domain and a cytoplasmic domain with serine/threonine kinase activity. T β RI also has an extracellular domain even though it does not bind TGF β when expressed without T β RII. The cytoplasmic portion of T β RI contains a kinase domain and a membrane proximal region which contains a Gly-Ser rich sequence (GS box) that has been proposed to be important for the activation of T β RI (10). Both receptors exist normally as homodimers on the cell surface (11, 12) and their kinase activities are essential for signal transduction (6, 8, 9, 13). Binding of TGF β 1 to T β RII induces the formation of a heteromeric complex of T β RI and T β RII (6, 8, 9, 13), which results in transphosphorylation of T β RI by the constitutively active T β RII (14). Phosphorylation of T β RI presumably activates its kinase activity and allows phosphorylation of downstream substrates such as Smad2 (15, 16, 17) and Smad3 (18). The phosphorylated Smad proteins then translocate to nucleus (18) and presumably, with the help of a DNA-binding molecule, activate downstream gene expression. Phosphorylation of serine residues at the carboxyl-terminal of Smad proteins is required for the nucleus translocation of Smad and subsequent growth inhibition. Mutations of these serine residues to alanine result in a Smad protein that is dominant negative in growth inhibition (16). The carboxyl half of Smad has transcriptional activating activity. Overexpression of this transactivating domain results in a Smad that is constitutively active in signal transduction (4, 19, 20).

As an initial step towards a better understanding of the multiple signaling pathways of the TGF β receptors, we have examined the regulation of the kinase activity and signal transduction of T β RII by autophosphorylation. We have shown that T β RII kinase is intricately regulated by autophosphorylation on at least three serine residues (21). Ser213, in the membrane-proximal segment outside the kinase domain, undergoes intra-molecular autophosphorylation which is

essential for the activation of T β RII kinase activity, activation of T β RI, and TGF β -induced growth inhibition. In contrast, phosphorylation of Ser409 and Ser416, located in the substrate recognition T-loop region in a three-dimensional structure model of protein kinases, is enhanced by receptor dimerization and can occur via an inter-molecular mechanism. Phosphorylation of Ser409 is essential for T β RII kinase signaling, while phosphorylation of Ser416 inhibits receptor function. Mutation of Ser416 to alanine results in a hyperactive receptor that is more able than wild-type to induce T β RI activation and subsequent cell cycle arrest. Both S409 and S416 are located in the substrate binding T-loop in the kinase domain and may involve in interaction with substrates.

In addition to these three sites, there is still another major site of autophosphorylation in T β RII whose identity was still not clear at the beginning of the fellowship period. This report focuses on the identification of the last major autophosphorylation site in T β RII and characterization of its function in signal transduction leading to growth inhibition. I also investigated downstream signaling events affected by autophosphorylation of T β RII.

Body

Experimental approach:

- 1. mapping the sites of autophosphorylation in T β RII:** To map the last major autophosphorylation sites in T β RII, Point mutations of individual serine residues in the cytoplasmic domain of T β RII were also carried out by PCR, and each mutant was analyzed by two-dimensional tryptic peptide mapping (21).
- 2. Analysis of the ability of T β RII containing mutations in the autophosphorylation sites to mediate growth inhibition:** Mutant receptors were stably introduced into the pro-B Ba/F3 cells as chimeric receptor with the extracellular and transmembrane domains of erythropoietin receptor (EpoR) and cytoplasmic domain of T β RII. Infected cells were incubated with various concentrations of Epo for 4 days and the growth of cells were measured by cell counting.
- 3. Analysis of the ability of mutant T β RII to transphosphorylate T β RI:** Ba/F3 cells containing mutant receptors were stimulated with or without Epo, and the receptor complex was isolated by immunoprecipitation. The ability of mutant T β RII to phosphorylate T β RI was measured in an in vitro kinase assay (21).
- 4. Analysis of the abilities of mutant T β RII to induce phosphorylation of Smad2:** Receptor complex containing wt and mutant T β RII and wt T β RI were isolated from transfected 293T cells by immunoprecipitation, and subjected to in vitro kinase assay in the presence of ^{32}P -ATP and purified Smad2 protein. Phosphorylated Smad2 was then resolved by SDS-PAGE.

Results and Discussions:

In addition to S213, S409 and S416, T β RII kinase undergoes autophosphorylation on S486.

Using mutagenesis analysis, I have identified the fourth major site of autophosphorylation to be S486 (Figure 1) which is located in the kinase domain. Mutation of S486 to alanine did not significantly affect the autophosphorylation activity of T β RII.

When compared in a three-dimensional structure of protein kinase, S486 is found to locate in a region unique to T β RII, indicating that this residue may have a regulatory function unique to T β RII. When compared with other kinases of the TGF β receptor family, S486 is also unique to T β RII and not present in T β RI and activin receptor or BMP receptor kinases.

Function of autophosphorylation on S486 in growth inhibition:

In contrast to S213, S409 and S416 whose phosphorylation affects kinase activity and signal transduction of T β RII significantly, mutation of S486 to alanine did not affect the kinase activity of T β RII, nor its ability to mediate growth inhibition. The growth of cells expressing the S486A mutant is indistinguishable from that of cells expressing the wt receptor (Figure 2).

Although S486 is not required for growth inhibition by T β RII, it may still be important for other aspects of T β RII signal transduction or regulation of T β RII activity. In addition to growth inhibition, TGF β can also induce gene activation and regulation of differentiation. Phosphorylation at S486 may play a role in the signaling activities leading to these functions. Receptor autophosphorylation has also been shown to be involved in receptor downregulation in the case of some tyrosine kinases. It is not clear whether receptor serine kinases employ similar mechanism of regulation. Since T β RII has very short half life on the cell surface (30 min), downregulation of T β RII is obviously a very important step of regulation. We will test the function of these autophosphorylated serine residues in the downregulation of T β RII.

Effects of T β RII autophosphorylation on the phosphorylation of Smad2: Since Smad2 has been shown to be a direct substrate of TGF β receptor complex and is required for signal transduction leading to growth inhibition, I decided to examine the effects of receptor autophosphorylation on the phosphorylation of Smad2 (Figure 3). Wild-type T β RII, when complexed with T β RI, can induce phosphorylation of Smad2 in an in vitro kinase assay. In contrast, receptor complex containing S213A failed to phosphorylate Smad2. This is consistent with the previous finding that S213A mutant is defective in kinase activity and can not transphosphorylate and activate T β RI. Therefore, the inactive receptor complex can not phosphorylate Smad2. Also consistent with the growth inhibition result, phosphorylation at S409 is also required for the phosphorylation of Smad2, since mutation of S409 to alanine failed to induce phosphorylation of Smad2. Although we have shown previously that S409A mutant is capable of transphosphorylating T β RI, this phosphorylation did not lead to activation of T β RI, as manifested by its inability to phosphorylate Smad2. This indicates that phosphorylation of T β RI does not always lead to activation of its kinase activity.

S416A mutant is hyperactive in promoting growth inhibition, and also induces a greater level of Smad2 phosphorylation. S486A mutant is indistinguishable from the wt receptor in its ability to mediate growth inhibition, and it can induce phosphorylation of Smad2 to a level similar to that induced by the wt T β RII. Therefore, the ability of these autophosphorylation mutants to induce Smad2 phosphorylation correlates perfectly with their abilities to mediate growth inhibition.

This again confirms the notion that Smad2 plays an important role in signal transduction leading to growth inhibition.

Autophosphorylation of T β RII occurs both intra- and inter-molecularly: To investigate whether autophosphorylation at S486 occurs via an intra- or inter-molecular mechanism, a kinase inactive T β RII was coexpressed in cells with the S486A mutant. If autophosphorylation at S486 occurs via an intra-molecular mechanism, after in vitro autophosphorylation, no phosphorylation can be observed, since the kinase inactive T β RII can not phosphorylate itself and S486A mutant no longer has the site to be phosphorylated. However, if autophosphorylation occurs via an inter-molecular mechanism, the kinase activity of S486A mutant could transfer the phosphate to the serine residue still present on the kinase inactive mutant.

I have found that upon cotransfection of the kinase inactive and S486A T β RII, peptide containing S486 was detected on a tryptic peptid map, suggesting that phosphorylation at S486 can occur via an inter-molecular mechanism.

The inter-molecular mechanism suggests an important function for a T β RII homodimer on the cell surface (11, 12). Despite the fact that T β RII homodimer alone is not sufficient for signal transduction, homodimerization of T β RII may play an important role in the regulation of T β RI activation. Dimerization of the RII cytoplasmic domain leads to increased phosphorylation of Ser409 or Ser416, which, in turn, modulates T β RI activation. Since phosphorylation of the two serine residues have opposite effects and since both are potential substrates for other cellular kinases, it is possible that, by regulating the ratio of phosphorylation at these two serines in response to ever-changing cellular environment, the extent of substrate phosphorylation can be fine-tuned in an accurate and reversible manner. In particular, the ratio of phosphorylation at Ser409 and Ser416 could alter in response to TGF β stimulation and this may provide a mechanism for the up or down regulation of receptor signal transduction.

Our results show that T β RII phosphorylation is intricately regulated and affects TGF β receptor signal transduction both positively and negatively. Therefore, the regulation of T β RII is not as simple as had been thought before. Multiple autophosphorylation events have been observed in receptor tyrosine kinases including PDGF receptor (22) and CSF-1 receptor (23). These phosphorylated tyrosine residues mediate interactions with different downstream signaling molecules and thus provide a mechanism that enables one receptor to initiate multiple signaling pathways. Since TGF β receptor can mediate complex biological responses, autophosphorylation on multiple serine residues may serve as a possible mechanism for the activation of different signal transduction pathways leading to these responses. Although identified as

autophosphorylation sites, these serine residues may also serve as targets for other cellular kinases, and thus the activity of T β RII could be up- or down-modulated in response to an array of intracellular signals. The identification of these important serine residues serves as a starting point to further dissect the regulatory mechanisms and signal transduction pathways of the TGF β receptors.

Figure 1. T β RII is autophosphorylated on S486. Mutant T β RII containing a point mutation changing S486 to A was isolated from transfected 293T cells by immunoprecipitation, subjected to autophosphorylation in vitro in the presence of γ 32P-ATP and analyzed by two dimensional tryptic peptide mapping. The arrow indicates the origin of loading. Spot c represent peptide containing S486.

Autophosphorylation at Ser486

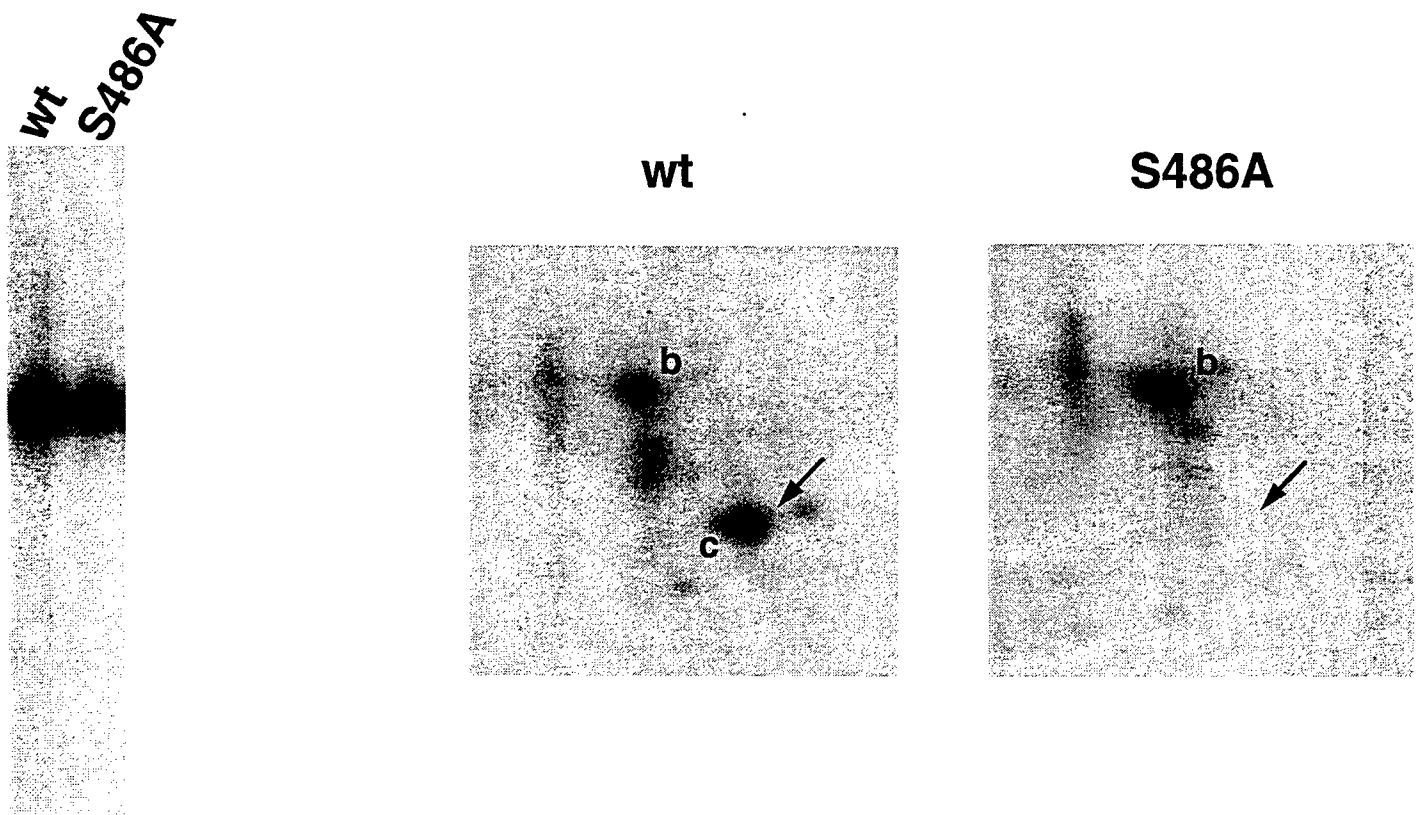


Figure 2: Growth inhibition assay

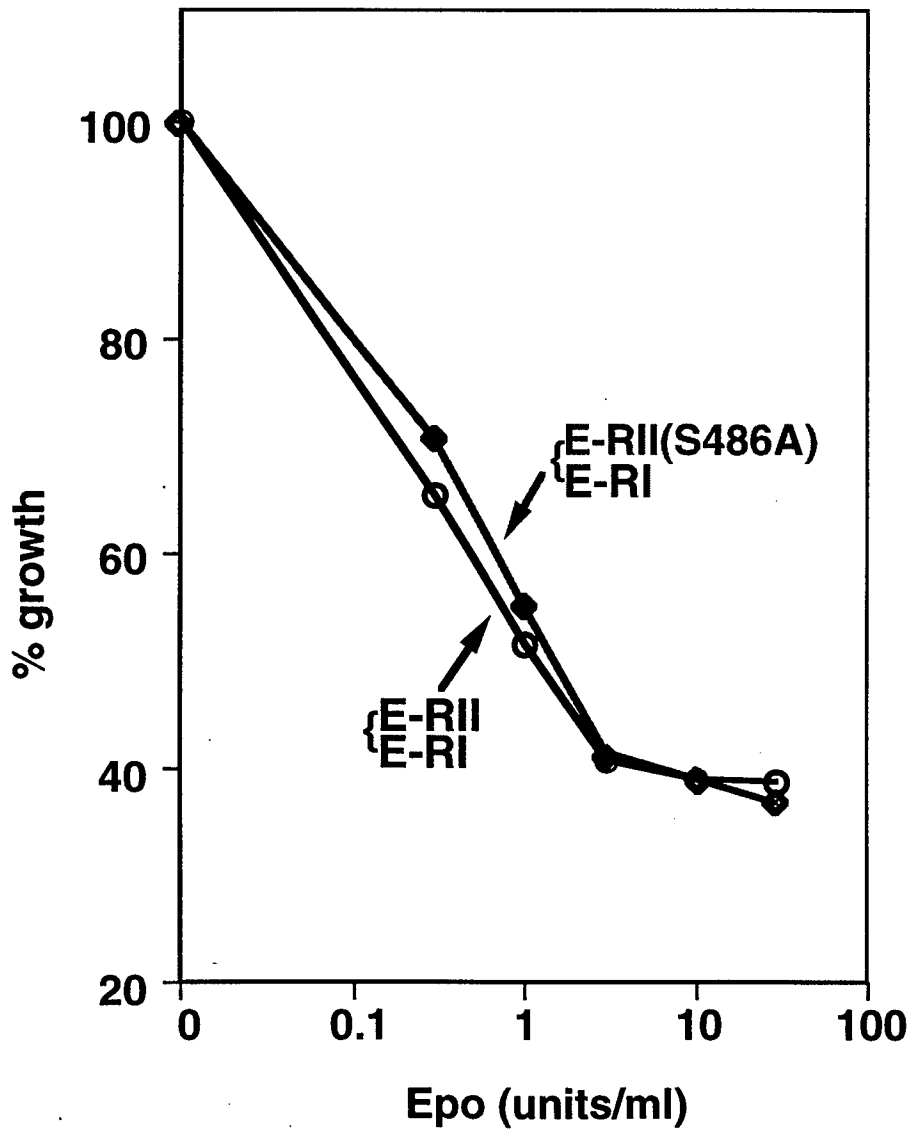


Figure 2. Growth inhibition assay. Ba/F3 cells expressing E-RII(S486A) and E-RI were incubated with increasing amount of Epo, and the growth of cells were examined by cell counting 4 days later.

Figure 3: Phosphorylation of Smad2 in vitro by mutant T β RIIs

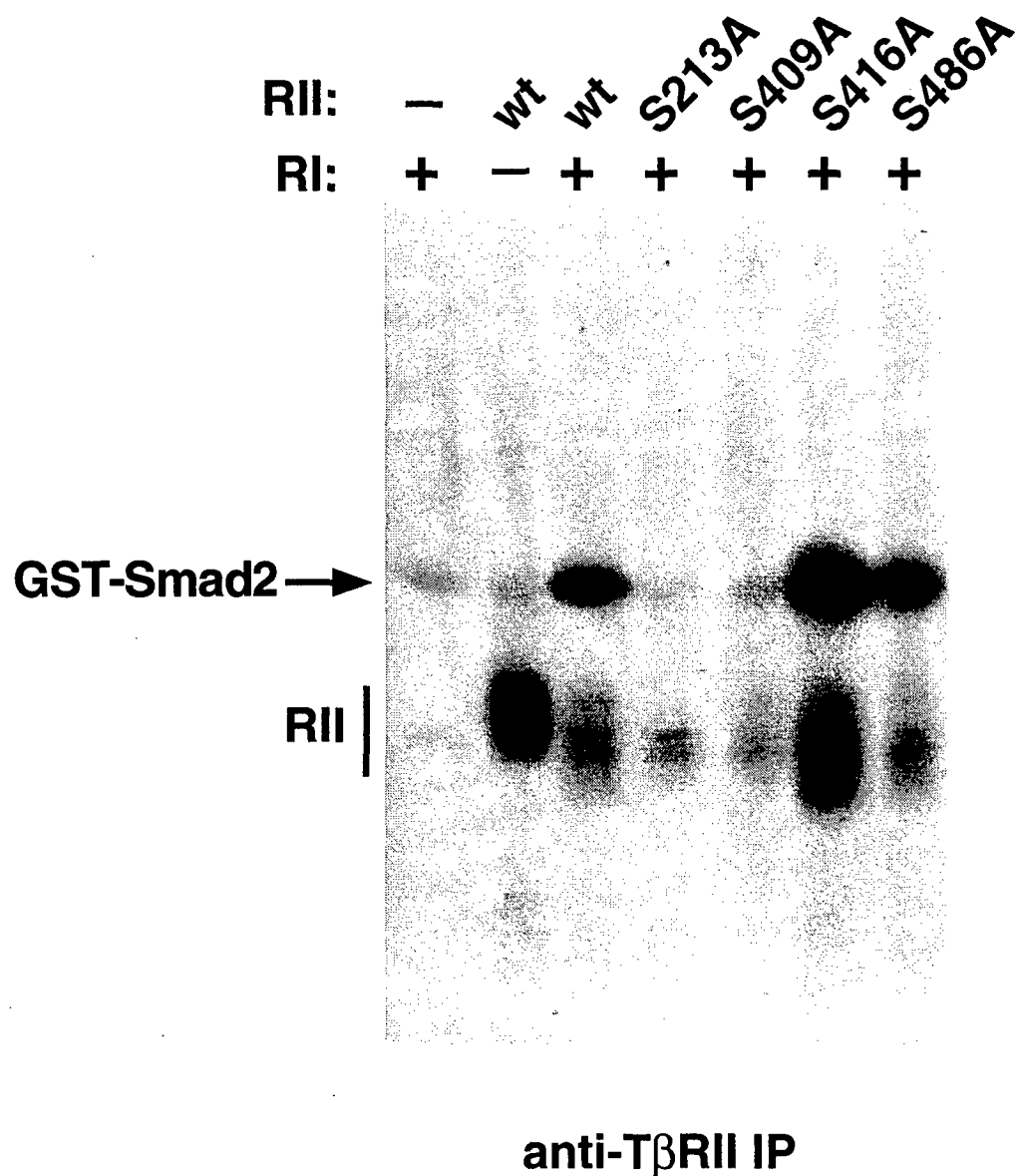


Figure 3. Phosphorylation of Smad2. Receptor complex containing wt E-RI and mutant E-RIIs were isolated from transfected 293T cells and incubated with 2 mg purified Smad2 in the presence of γ -³²P-ATP.

Conclusion:

Combined with our previous studies, the studies presented here showed for the first time that T β RII kinase is regulated intricately by autophosphorylation on multiple sites. The identification of these site will no doubt help us dissect the complex signaling system of the TGF β receptors and also provide a starting point for the isolation of interacting downstream signaling molecules. It also provides a handle to start understanding the mechanism of regulation for the activation of TGF β receptors.

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